

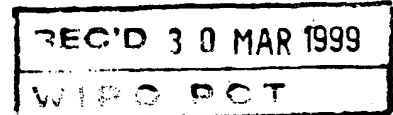


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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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## PRIORITY DOCUMENT

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Title: Novel molecules involved in apoptotic pathways.

The present invention relates to the field of apoptosis, as well as to the field of cancer diagnosis and treatment, and treatment and diagnosis of (auto-)immune diseases and other diseases related to regulation of apoptosis. The present invention specifically relates to molecules found to be involved in apoptotic pathways and their uses in inducing apoptosis in aberrant cells. The presently invented molecules have been identified using apoptin. Apoptin is a protein originally found in chicken anemia virus (CAV; Noteborn et al., 1991) and was originally called VP3. The apoptotic activity of this protein was discovered by the group of the present inventors (Noteborn et al., 1994).

Apoptosis is an active and programmed physiological process for eliminating superfluous, altered or malignant cells (Earnshaw, 1995, Duke et al., 1996). Apoptosis is characterized by shrinkage of cells, segmentation of the nucleus, condensation and cleavage of DNA into domain-sized fragments, in most cells followed by internucleosomal degradation. The apoptotic cells fragment into membrane-enclosed apoptotic bodies. Finally, neighbouring cells and/or macrophages will rapidly phagocytose these dying cells (Wyllie et al., 1980, White, 1996). Cells grown under tissue-culture conditions and cells from tissue material can be analysed for being apoptotic with agents staining DNA, as e.g. DAPI, which stains normal DNA strongly and regularly, whereas apoptotic DNA is stained weakly and/or irregularly (Noteborn et al., 1994, Telford et al., 1992).

The apoptotic process can be initiated by a variety of regulatory stimuli (Wyllie, 1995, White 1996, Levine, 1997). Changes in the cell survival rate play an important role in human pathogenesis, e.g. in cancer development and auto-immune diseases, which is caused by enhanced proliferation but also by decreased cell death (Kerr et al., 1994,

Paulovich, 1997). A variety of chemotherapeutic compounds and radiation have been demonstrated to induce apoptosis in tumor cells, in many instances via wild-type p53 protein (Thompson, 1995, Bellamy et al., 1995, Steller, 1995, McDonnell et al., 1995).

Many tumors, however, acquire a mutation in p53 during their development, often correlating with poor response to cancer therapy. Transforming genes of tumorigenic DNA viruses can inactivate p53 by directly binding to it (Teodoro, 1997). An example of such an agent is the large T antigen of the tumor DNA virus SV40. For several (leukemic) tumors, a high expression level of the proto-oncogene Bcl-2 or Bcr-abl is associated with a strong resistance to various apoptosis-inducing chemotherapeutic agents (Hockenberry 1994, Sachs and Lotem, 1997).

For such cancers (representing more than half of the tumors) alternative anti-tumor therapies are under development based on induction of apoptosis independent of p53 (Thompson 1995, Paulovich et al., 1997). One has to search for the factors involved in induction of apoptosis, which do not need p53 and/or can not be blocked by Bcl-2/Bcr-abl-like anti-apoptotic activities. These factors might be part of a distinct apoptosis pathway or might be (far) downstream to the apoptosis inhibiting compounds.

Apoptin is a small protein derived from chicken anemia virus (CAV; Noteborn and De Boer, 1995, Noteborn et al., 1991, Noteborn et al., 1994), which can induce apoptosis in human malignant and transformed cell lines, but not in untransformed human cell cultures. In vitro, apoptin fails to induce programmed cell death in normal lymphoid, dermal, epidermal, endothelial and smooth-muscle cells. However, when normal cells are transformed they become susceptible to apoptosis by apoptin. (Danen-van Ooschot, 1997 and Noteborn, 1996). Long-term expression of apoptin in normal human fibroblasts revealed that apoptin has no toxic or transforming activity in these cells.

In normal cells, apoptin was found predominantly in the cytoplasm, whereas in transformed or malignant cells i.e. characterized by hyperplasia, metaplasia or dysplasia, it was located in the nucleus, suggesting that the localization of apoptin is related to its activity (Danen-van Oorschot et al. 1997).

Apoptin-induced apoptosis occurs in the absence of functional p53 (Zhuang et al., 1995a), and cannot be blocked by Bcl-2, Bcr-abl (Zhuang et al., 1995), the Bcl-2-associating protein BAG-1 and not by the caspase-inhibitor cowpox protein CrmA (Danen-Van Oorschot, 1997a, Noteborn, 1996).

Therefore, apoptin is useful for the destruction of tumor cells, or other hyperplasia, metaplasia or dysplasia which have become resistant to (chemo)therapeutic induction of apoptosis, due to the lack of functional p53 and (over)-expression of Bcl-2 and other apoptosis-inhibiting agents (Noteborn et al., 1997).

The fact that apoptin does not induce apoptosis in normal human cells, at least not in vitro, suggests that a toxic effect of apoptin treatment in vivo will be very low. Noteborn et al. (1997) have provided evidence that adenovirus expressed apoptin does not have an acute toxic effect in vivo. In addition, in nude mice it was shown that apoptin has a strong anti-tumor activity.

It appears, that even pre-malignant, minimally transformed cells, are sensitive to the death-inducing effect of apoptin. In addition, Noteborn and Zhang (1997) have shown that apoptin-induced apoptosis can be used as diagnosis of cancer-prone cells and treatment of cancer-prone cells.

Knowing that apoptin is quite safe in normal cells, but that as soon as a cell becomes transformed and/or immortalized (the terms may be used interchangeable herein) the present inventors designed some uses based on the identification of compounds involved in the apoptin-induced apoptotic cascade. These compounds are factors of an

apoptosis pathway, which is specific for transformed cells. Therefore, these proteins are very important compounds in new treatments and diagnosis for diseases related with aberrancies in the apoptotic process, such as cancer and  
5 auto-immune diseases.

Proteins found associating with apoptin include members of the family of Nmi/Hou-like and IFP-like proteins.

Thus the invention provides a recombinant and/or isolated nucleic acid molecule encoding at least a functional  
10 part of a member of the family of Nmi-like proteins or at least a functional part of a member of the family of Hou-like proteins or at least a functional part of a member of the family of IFP35-like proteins for use in the induction of apoptosis in a population of cells related to a pathological  
15 condition.

As explained herein the expression of Hou is connected to oncogenes and has been found to be high in certain transformed cells. These are typically the cells that can be induced to go into apoptosis by apoptotic agents such as  
20 apoptin. Typically providing a cell with Hou-like activity will therefor increase the chance of inducing apoptosis in such a cell. IFP35-like proteins are involved in transporting apoptotic substances to the nucleus of cells. Under influence of for instance interferons these proteins localize in the  
25 nucleus. Therefor IFP-like activity is used to get apoptin-like activity into the nucleus, which is important for the induction of apoptosis, for instance through Hou-like proteins. The Hou-like activity or Nmi-like activity is defined herein as any molecule capable of exerting the same  
30 or a similar function as the original Hou-like (Nmi-like) protein. The same definition goes for IFP-activity. Typically such a molecule can be encoded by a nucleic acid molecule which comprises at least a functional and specific part of the sequence of figure 1, 2, 4 or 5 or encoding an amino  
35 sequence of figure 6 or a sequence at least 60, preferably 70, preferably 90 % homologous with said functional and

specific sequence or comprising a sequence hybridizing to any of the foregoing sequences under stringent conditions. In order to be able to express the Hou-like activity and/or the IFP-like activity it is preferred to have an expression  
5 vector encoding said activity. Expression vectors are nucleic acid molecules which can be brought into cells, or transfect cells themselves and which have the machinery (together with the machinery of the host cell) to express proteins encoded on the expression vector when present in a cell.

10 It is preferred that cells which are provided, according to the invention, with Hou-like activity and/or IFP-like activity, are also provided with apoptosis inducing activity, preferably apoptin-like activity, which is defined along the same lines as Hou-like activity. In order to get the activity  
15 into the cells in which apoptosis has to be induced it is possible and preferred to use a gene delivery vehicle. A gene delivery vehicle is a means to transport a nucleic acid molecule capable of expressing the wanted activity in a host cell into said host cell. Gene delivery vehicles are known in  
20 the art. They include for instance recombinant viruses such as adenoviruses and retroviruses, but also non-viral vehicles such as polymers and liposomes have been suggested. Methods of targeting gene delivery vehicles to target cells are also known in the art and need not be elaborated herein. The  
25 invention also provides the newly identified molecules themselves, both the nucleic acid molecules (meaning DNA coding and/or non coding strands as well as RNA) and the proteinaceous molecules (peptides, polypeptides, glycoproteins and associations between proteins and RNA's and  
30 the like). Based on the given sequences other family members of the Hou/Nmi and IFP families will be identified having the same or similar function. Typically such molecules will have high homology to the sequences given herein.

For nucleic acid molecules the homology is expected to  
35 be at least 60, preferably 70, more preferably 80%.  
therewith.

These nucleic acid molecules can of course again be incorporated into expression vectors as mentioned hereinbefore. Preferably these expression vectors also encode apoptotic activity, preferably apoptin or a functional  
5 fragment and/or equivalent thereof.

These expression vectors can again be made into gene delivery vehicles.

The invention also provides the recombinant or isolated proteinaceous substance comprising at least a functional part  
10 of a member of the family of Nmi/Hou-like proteins or at least a functional part of a member of the family of Hou-like proteins for use in the induction of apoptosis in a population of cells related to a pathological condition and an Nmi/Hou-like proteinaceous substance having at least a  
15 functional and/or specific part of the sequence of figure 3 or being encoded by a functional and/or specific part of the sequence of figure 1 or figure 2 or being at least 60, preferably 70, preferably 80% homologous to at least a functional and/or specific part of the sequence of figure 3  
20 or being at least 60, preferably 70, preferably 80% homologous to a protein encoded by at least a functional and/or specific part of the sequence of figure 1 or figure 2 and an IFP35-like proteinaceous substance having at least a functional and/or specific part of the sequence of figure 6  
25 or 7 or being encoded by a functional and/or specific part of the sequence of figure 4 or figure 5 or being at least 60, preferably 70, preferably 80% homologous to at least a functional and/or specific part of the sequence of figure 6 or 7 or being at least 60, preferably 70, preferably 80%  
30 homologous to a protein encoded by at least a functional and/or specific part of the sequence of figure 4 or figure 5.

A functional part in this document means having the same or similar activity (although the amount of activity may differ). A specific part herein means a part of sufficient  
35 size to be specific for the protein or nucleic acid or to be of sufficient size to distinguish the protein from another



protein immunologically. The proteins disclosed herein can for instance also be used to identify further components of the apoptotic pathway.

5 The reason for bringing IFP-like activity and/or Hou-like activity together with apoptotic activity is of course to induce aberrant cells to go into apoptosis. Thus the invention also provides a method for inducing apoptosis in cells comprising providing said cells with Nmi/Hou-like protein activity and/or IFP-35-like activity together with  
10 apoptin-like activity.

The invention further provides a method for inducing apoptosis through interference with the function of Nmi/Hou-like proteins (interchangeably referred as Hou-, Nmi- or Nmi/Hou-like proteins).

15 The invention provides an anti-tumor therapy based on the interference with the function of Hou or Hou-like proteins. The fact that Hou or Hou-like proteins are abundantly present in tumor cells in combination with highly expressed oncogenes, - which are activated by Hou or Hou-like  
20 proteins -, make Hou and/or Hou-like proteins very important targets of an anti-tumor agent/therapy.

The invention provides the mediator of apoptin-induced apoptosis, which is tumor-specific.

25 The invention provides a therapy for cancer, auto-immune diseases or related diseases which is based on Hou-like proteins in combination with apoptin and/or apoptin-like compounds.

30 The invention further provides a method for inducing apoptosis through interference with the function of IFP35-like proteins.

The invention provides an anti-tumor therapy based on the interference with the function of IFP35 or IFP35-like proteins.

35 The invention provides IFP35 as a mediator of apoptin-induced apoptosis, which is tumor-specific.

The invention provides a therapy for cancer, auto-immune diseases or related diseases which is based on IFP35 or IFP35-like proteins in combination with apoptin and/or apoptin-like compounds.

5       The invention provides a therapy based on the combination of apoptin-associating proteins Hou and IFP35.

The invention further provides a method for inducing apoptosis through interference with the function of IFP35 and Hou or IFP35-and Hou-like proteins.

10       The invention provides an anti-tumor therapy based on the interference with the function of IFP35 or IFP35-like proteins, in combination with Hou or Hou-like proteins.

The invention provides IFP35 or IFP35-like in combination with Hou or Hou-like proteins as mediators of  
15       apoptin or apoptin-like induction of apoptosis, which is tumor-specific.

The invention provides a therapy for cancer, auto-immune diseases or related diseases which is based on the combination of IFP35 or IFP35-like and Hou or Hou-like  
20       proteins in combination with apoptin and/or apoptin-like compounds.

Furthermore, the invention provides a diagnosis, based on Hou or Hou-like proteins, which can determine whether cells are susceptible for apoptin-induced apoptosis. This  
25       means that patients with tumors having up-regulated Hou or Hou-like activity are optimal candidates for an anti-cancer therapy based on apoptin.

Furthermore, the invention provides a diagnosis, based on Hou or Hou-like proteins in combination with IFP35 or  
30       IFP35-like proteins, which can determine whether cells are susceptible for apoptin-induced apoptosis. This means that patients with tumors having up-regulated Hou or Hou-like activity in combination with IFP35 or IFP35-like activity are optimal candidates for an anti-cancer therapy based on  
35       apoptin.

The invention will be explained in more detail in the following experimental part. This only serves for the purpose of illustration and should not be interpreted as a limitation of the scope of the invention.

## EXPERIMENTAL PART

The inventors have used the yeast-2 hybrid system (Durfee et al., 1993) to identify apoptin-associating cellular compounds, which are essential in the induction of apoptosis. The used system is an in vivo strategy to identify human proteins capable of physically associating with apoptin. It has been used to screen cDNA libraries for clones encoding proteins capable of binding to a protein of interest (Fields and Song, 1989, Yang et al., 1992).

### Construction of pGBT9-VP3

For the construction of the bait plasmid, which enables the identification of apoptin-associating proteins by means of a yeast-two-hybrid system, plasmid pET-16b-VP3 (Noteborn, unpublished results) was treated with NdeI and BamHI. The 0.4 kb NdeI-BamHI DNA fragment was isolated from low-melting-point agarose.

Plasmid pGBT9 (Clontech Laboratories, Inc, Palo Alto, USA) was treated with the restriction enzymes EcoRI and BamHI. The about 5.4-kb DNA fragment was isolated and ligated to an EcoRI-NdeI linker and the 0.4-kb DNA fragment containing the apoptin-encoding sequences starting from its own ATG-initiation codon. The final construct containing a fusion gene of the GAL4-binding domain sequence and apoptin under the regulation of the yeast promoter ADH was called pGBT-VP3 and was proven to be correct by restriction-enzyme analysis and DNA-sequencing according to the Sanger method (1977).

All cloning steps were essentially carried out as described by Maniatis et al. (1992). The plasmid pGBT-VP3 was purified by centrifugation in a CsCl gradient and column chromatography in Sephacryl S500 (Pharmacia).

### GAL4-activation domain-tagged cDNA library

The expression vector pACT, containing the cDNAs from Epstein-Barr-virus-transformed human B cells fused to the

GAL4 transcriptional activation domain, was used for detecting apoptin-associating proteins. The pACT c-DNA library is derived from the lambda-ACT cDNA library, as described by Durfee et al. 1993.

5

#### Bacterial and Yeast strains

The E.coli strain JM109 was the transformation recipient for the plasmid pGBT9 and pGBT-VP3. The bacterial strain electromax/DH10B was used for the transformation needed for the recovery the apoptin-associating pACT-cDNAs, and was obtained from GIBCO-BRL, USA.

The yeast strain Y190 was used for screening the cDNA library, and all other transformations which are part of the used yeast-two-hybrid system.

15

#### Media

For drug selections Luria Broth (LB) plates for E.coli were supplemented with ampicillin (50 microgram per ml). Yeast YPD and SC media were prepared as described by Rose et al. (1990).

20

#### Transformation of competent yeast strain Y190 with plasmids pGBT-VP3 and pACT-cDNA and screening for beta-galactosidase activity.

The yeast strain Y190 was made competent and transformed according to the methods described by Klebe et al. (Klebe et al., 1983). The yeast cells were first transformed with pGBT-VP3 and subsequently transformed with pACT-cDNA, and these transformed yeast cells were grown on histidine-minus plates, also lacking leucine and tryptophan.

30

Hybond-N filters were layed on yeast colonies, which were histidine-positive and allowed to wet completely. The filters were lifted and submerged in liquid nitrogen to permeabilize the yeast cells. The filters were thawed and layed with the colony side up on Whattman 3MM paper in a petridish with Z-buffer (Per liter: 16.1 gr  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 5.5

35

gr  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 0.75 gr KCl and 0,246 gr  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , pH 7.0) containing 0.27% beta-mercapto-ethanol and 1 mg/ml X-gal. The filters were incubated for at least 15 minutes or during night.

5

#### Recovery of plasmids from yeast

Total DNA from yeast cells, which were histidine- and beta-galactosidase-positive, was prepared by using the glusulase-alkaline lysis method as described by Hoffman and Winston (1987) and used to transform Electromax/DH10B bacteria via electroporation using a Bio-Rad GenePulser according the manufacturer's specifications.

Transformants were plated on LB media containing ampicillin.

15

#### Isolation of apoptin-associating pACT clones

By means of colony-filter assay the colonies were lysed and hybridized to a radioactive-labeled 17-mer oligomer, which is specific for pACT (see also section Sequence analysis).

Plasmid DNA was isolated from the pACT-clones, and by means of XhoI digestion analysed for the presence of a cDNA insert.

#### 25 Sequence analysis

The subclones containing the sequences encoding apoptin-associating proteins were sequenced using dideoxy NTPs according to the Sanger method which was performed by Eurogentec, Nederland BV (Maastricht, The Netherlands). The used sequencing primer was a pACT-specific 17-mer comprising of the DNA-sequence 5'-TACCACTACAATGGATG-3'.

The sequences of the apoptin-associating proteins were compared with known gene sequences from the EMBL/Genbank.

## Results and discussion

Apoptin induces specifically apoptosis in transformed cells, such as cell lines derived from human tumors. To identify the essential compounds in this cell-transformation-specific and/or tumor-specific apoptosis pathway, a yeast  
5 genetic screen was carried out.

We have used a human cDNA library, which is based on the plasmid vector pACT containing the complete cDNA copies made from Epstein-Barr virus-transformed human B cells (Durfee et  
10 al., 1993).

### Construction of a bait plasmid expressing a fusion gene product of GAL4-DNA-binding domain and apoptin

To examine the existence of apoptin-associating proteins  
15 in the human transformed/tumorigenic cDNA library, a so-called bait plasmid had to be constructed.

To that end, the complete apoptin-encoding region, flanked by about 40 basepairs downstream from the apoptin gene, was cloned in the multiple cloning site of plasmid  
20 pGBT9.

The final construct, called pGBT-VP3, was analysed by restriction-enzyme analysis and sequencing of the fusion area between apoptin and the GAL4-DNA-binding domain.

25 A gene(fragment) encoding an apoptin-associating protein is determined by transactivation of a GAL4-responsive promoter in yeast.

The apoptin gene is fused to the GAL4-DNA-binding domain of plasmid pGBT-VP3, whereas all cDNAs derived from the  
30 transformed human B cells are fused to the GAL4-activation domain of plasmid pACT. If one of the cDNAs will bind to apoptin, the GAL4-DNA-binding domain will be in the vicinity of the GAL4-activation domain resulting in the activation of the GAL4-responsive promoter, which regulates the reporter  
35 genes HIS3 and LacZ.

The yeast clones containing plasmid expressing apoptin and a plasmid expressing an apoptin-associating protein fragment can grow on a histidine-minus medium and will stain blue in a beta-galactosidase assay. Subsequently, the plasmid  
5 with the cDNA insert encoding the apoptin-associating protein can be isolated and characterized.

Before we could do so, however, we have determined that transformation of yeast cells with pGBT-VP3 plasmid alone or in combination with an empty pACT vector, did not result in  
10 the activation of the GAL4-responsive promoter.

#### Identification of apoptin-associating proteins encoded by cDNAs derived from a human transformed B cell line.

We have found yeast colonies, which upon transformation  
15 with pGBT-VP3 and pACT-cDNA were able to grow on a histidine-minus medium (also lacking leucine and tryptophan) and stained blue in a beta-galactosidase assay. These results indicate that the observed yeast colonies contain besides the bait plasmid pGBT-VP3 also a pACT plasmid encoding a  
20 potential apoptin-associating protein.

Plasmid DNA was isolated from these positive yeast colonies, which were transformed in bacteria. By means of a filter-hybridization assay using a pACT-specific labeled DNA-probe, the clones containing pACT plasmid could be  
25 determined. Subsequently, pACT DNA was isolated and digested with restriction enzyme XhoI, which is indicative for the presence of a cDNA insert. Finally, the pACT plasmids with a cDNA insert were sequenced.

#### 30 Description of apoptin-associating proteins

The yeast genetic screen for apoptin-associating proteins resulted in the detection of two types of proteins, namely a Hou/Nmi-like protein and an IFP35-like protein. The apoptin-associating amino-acid sequences are homologous with  
35 the known Hou/Nmi amino-acid sequence or homologous with the



known IFP35 amino-acid sequence. Hou/Nmi also share a homologous region (see below).

The determined DNA sequences of the two independent Hou/Nmi cDNA clones are shown in Figures 1 and 2, respectively. The amino acid sequence, derived from the detected DNA sequences is given in Figure 3. Remarkably, the complete open-reading frame (ORF) of the Hou-like protein was proven to be characterized.

The found DNA sequences of the three independent IFP35-like cDNA clones are shown in Figures 4, 5 and 6, respectively. Figure 7 shows the combination of the 2 independent IFP35 amino-acid sequences. The common part of these clones will associate with apoptin.

Interestingly, the C-terminus of Nmi shows homology to IFP35 (a.a. 102-288, 46% similarity; (Bao and Zervos, 1996, Bange et al, 1994). Actually, these data show that it is expected that our genetic yeast screen has resulted in these two apoptin-associating proteins, for they share a common homologous region.

20

#### **Hou/Nmi-like proteins**

The remarkable feature of apoptin-induced apoptosis is its tumor-specific activity. The fact that apoptin binds to Hou/Nmi-like proteins unravels this tumor/transformation-specific activity of apoptin. Below, the terms Hou/Nmi-like, Nmi, or Hou will be interchangeably used.

In this respect, the pattern of Nmi expression is interesting, since it is expressed at low levels in normal tissues, in contrast to its high levels of expression in transformed cell lines. Among eight cancer lines tested, highest levels were observed in four leukemia cell lines (Bao and Zervos, 1996).

In leukemias, a high expression of C-myc correlates with a high level of Nmi (HL-60, K562 and MOLT-4). The Nmi gene is located on chromosome 22, which is also involved in the t(9;22) translocation leading to the Bcr-Abl fusion protein,

as seen in some leukemias (Rabbits, 1991, Sawyers and Deny, 1994).

Using a yeast genetic screen, Nmi was identified as a protein that binds to N-myc and C-myc. Myc proteins are important in the regulation of cell proliferation and differentiation. Together with ras or raf, myc can transform primary cells in culture. Nmi/Hou-like proteins will up-regulate the activity of Myc proteins via binding to them.

Up-regulation of Myc proteins has been described for Burkitt lymphomas, neuroblastomas and small cell lung carcinomas. Myc proteins contain a basic region, a helix-loop-helix (HLH) and a leucine zipper (Zip), and form homo- or heterodimers that can bind to specific DNA sequences and regulate transcription. Myc also forms heterodimers with Max. Myc/Max heterodimers activate transcription, whereas Max homodimers repress transcription, thus antagonizing Myc's function (Evan and Littlewood, 1993).

Nmi was found to interact with N-myc, c-myc, Max, Mxi1 and other transcription factors that have HLH and/or Zip motifs. Interaction with N-myc and C-myc was confirmed by co-precipitation experiments (Bao and Zervos, 1996).

#### **Induction of apoptosis through interference with the function of Nmi/Hou-like proteins.**

Our results indicate that apoptin can change the Nmi/Hou-like-mediated proliferation (transformation/tumor-formation) activity into a Nmi/Hou-like-mediated apoptotic activity. Remarkably, this Nmi/Hou-like-mediated apoptotic activity will be specific for transformed/tumor cells, due to the very high level of Nmi/Hou in transformed cells in combination with over-expression of (proto-)oncogenes, such as Myc.

By means of transient transfection assays, it was shown that over-expression of the determined Hou-like protein (see Fig. 3) and apoptin did result in induction of apoptosis in normal VH10-, VH25-fibroblasts. In contrast to normal

fibroblasts which over-expressed only apoptin. This result indicates that Hou-like proteins are an important factor in (apoptin-induced) apoptosis.

The presented data imply that interference with the function of Nmi/Hou-like proteins resulting in apoptosis can be used as a specific anti-tumor therapy, or therapies of related diseases, such as auto-immune diseases.

#### **Characteristics of the apoptin-associating protein IFP35**

The other apoptin-associating protein is IFP35, which is an interferon(IFN)-induced leucine zipper protein of 282 a.a., and has an apparent molecular mass of 35 kD. It was isolated by differential screening from HeLa cells that had been treated with IFN- $\gamma$  (Bange et al., 1994).

IFP35 mRNA could be induced by IFN- $\gamma$  in different human cell types, including fibroblasts, macrophages, and epithelial cells. It has a leucine zipper motif at the N-terminus, but it lacks an adjacent basic domain required for DNA binding. It has been suggested that these types of proteins negatively regulate bZIP transcription factors by forming non-functional heterodimers. IFP35 was shown to form homodimers (Bange et al., 1994).

#### **Induction of apoptosis by interference of IFP35 in combination with Hou/Nmi-like proteins.**

IFP35 is found in the cell nucleus, after interferon treatment and is expressed in a wide variety of cell types including fibroblasts, macrophages and epithelial cells (Bange et al., 1994).

In general, virus infections trigger interferon production. It is likely that a CAV infection and/or expression of apoptin will result in interferon up-regulation, which might result in the translocation of IFP35 or IFP35-like proteins into the nucleus. IFP35 will transport apoptin also to the nucleus, due to its association.

It seems likely that if apoptin is transported into the nucleus by IFP35 it will be able to associate with the IFP35-homologous region within Hou/Nmi-like proteins. This association will cause an aberrant regulation of Hou/Nmi-regulated genes, such as the oncogene Myc. Subsequently, the cells over-expressing Nmi/Hou-like proteins and oncogenes, such as Myc will undergo apoptosis.

Experimental evidence for IFP35 as an essential factor in (apoptin) apoptosis induction was derived from the following experiments. Normal VH10 cells over-expressing Hou/Nmi, IFP35 and apoptin underwent faster apoptosis than normal VH10 cells expressing Hou/Nmi and apoptin.

### Conclusions

In conclusion, we have provided evidence that interference of specific factors with the function of Nmi/Hou, of IFP35 or of both will result in induction of apoptosis.

Therapies based on induction of apoptosis are possible if they succeed in the interference with the function of Nmi/Hou-like and/or of IFP35-like proteins. An example of such an interfering factor is apoptin. Another CAV-derived protein, which is known to induce apoptosis and also known to enhance apoptin activity is VP2 (Noteborn et al., 1997).

### Other apoptin-associating proteins

The genetic yeast screen with pGBT-VP3 as bait plasmid and pACT plasmid containing cDNAs from transformed human B cells also delivered the protein filamin. The protein filamin is localized within lamellipodia and filopodia. Filamin is one of the cross-linking proteins of actin. It may play an additional role of linking the cytoskeleton to cell-substratum adhesion sites (Matsudaira, 1994). Two independent filamin-like clones were found. The found associating amino acid sequence of the two filamin clones are shown in Figure 8.

### Description of the figures

Figure 1 shows the DNA sequence of the analysed region of the apoptin-associating clone Nmi/Hou-like No-1.

5

Figure 2 shows the DNA sequence of the analysed region of the apoptin-associating clone Nmi/Hou-like No-2.

Figure 3 shows the combination of the amino acids of the sequenced Nmi/Hou-like, derived from clones No-1 and No-2. In addition, the three C-terminal amino acids H-E-G of the multiple cloning site of pACT are given to illustrate that the Nmi/Hou-like amino acid sequence is in frame with the GAL4-activation domain. This feature proves that the Nmi/Hou-like region is indeed synthesized in yeast cells.

10  
15

Figure 4 shows the DNA sequence of the analysed region of the apoptin-associating clone IFP35-like No-1.

Figure 5 shows the DNA sequence of the analysed region of the apoptin-associating clone IFP35-like No-2.

Figure 6 shows the amino acids of the sequenced region of the apoptin-associating clone IFP35-like No-3.

25

Figure 7 shows the combination of the amino acids of the sequenced IFP35-like clones No-2 and No-3. The fact that they overlap with each other implies that the common region of these three inserts associates with apoptin. In addition, part of the amino acid sequence of the known IFP35 is shown.

30

Figure 8 shows the amino acids of the sequenced region of the apoptin-associating clone Filamin No-1 and No-2. In addition, the three C-terminal amino acids H-E-G of the multiple cloning site of pACT are given to illustrate that the filamin-like amino acid sequence is in frame with the GAL4-

35

activation domain. This feature proves that the filamin-like region is indeed synthesized.

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CLAIMS

1. A recombinant and/or isolated nucleic acid molecule encoding at least a functional part of a member of the family of Nmi-like proteins or at least a functional part of a member of the family of Hou-like proteins or at least a functional part of a member of the family of IFP35-like proteins for use in the induction of apoptosis in a population of cells related to a pathological condition.
2. A use according to claim 1 wherein said nucleic acid molecule comprises at least a functional and specific part of the sequence of figure 1, 2, 4 or 5 or encoding an amino sequence of figure 6 or a sequence at least 60, preferably 70, preferably 90 % homologous with said functional and specific sequence or comprising a sequence hybridizing to any of the foregoing sequences under stringent conditions.
3. Use according to claim 1 or 2 wherein said nucleic acid comprises an expression vector.
4. Use according to anyone of the foregoing claims whereby said cells are provided with apoptosis inducing activity.
5. Use according to claim 4 whereby said apoptosis inducing activity is apoptin-like activity.
6. Use according to any of claims 1-5 wherein said nucleic acid is part of a gene delivery vehicle.
7. A recombinant and/or isolated nucleic acid molecule encoding an Nmi/Hou-like protein comprising at least a functional and/or specific part of the sequence of figure 1 or figure 2 or a sequence at least 60, preferably 70, more preferably 80% homologous therewith.
8. A recombinant and/or isolated nucleic acid molecule encoding an IFP35-like protein comprising at least a functional and/or specific part of the sequence of figure 4 or figure 5 or encoding at least a functional and/or specific part of the amino acid sequence of figure 6 or a sequence at least 60, preferably 70, more preferably 80% homologous therewith.

9. An expression vector comprising a recombinant nucleic acid molecule according to claim 7 and/or 8.
10. An expression vector according to claim 9 further comprising a sequence encoding apoptotic activity.
- 5 11. An expression vector according to claim 10 wherein said sequence encoding apoptotic activity encodes apoptin or a functional fragment and/or equivalent thereof.
12. A gene delivery vehicle comprising a recombinant nucleic acid molecule according to claim 7 or 8 or an expression
- 10 vector according to anyone of claims 9-11.
13. A recombinant or isolated proteinaceous substance comprising at least a functional part of a member of the family of Nmi/Hou-like proteins or at least a functional part of a member of the family of Hou-like proteins for use
- 15 in the induction of apoptosis in a population of cells related to a pathological condition.
14. An Nmi/Hou-like proteinaceous substance having at least a functional and/or specific part of the sequence of figure 3 or being encoded by a functional and/or specific part of the
- 20 sequence of figure 1 or figure 2 or being at least 60, preferably 70, preferably 80% homologous to at least a functional and/or specific part of the sequence of figure 3 or being at least 60, preferably 70, preferably 80% homologous to a protein encoded by at least a functional
- 25 and/or specific part of the sequence of figure 1 or figure 2.
15. A recombinant or isolated proteinaceous substance comprising at least a functional part of a member of the family of Nmi/Hou-like proteins or at least a functional part of a member of the family of Hou-like proteins for use
- 30 in the induction of apoptosis in a population of cells related to a pathological condition.
16. An IFP35-like proteinaceous substance having at least a functional and/or specific part of the sequence of figure 6 or 7 or being encoded by a functional and/or specific part of
- 35 the sequence of figure 4 or figure 5 or being at least 60, preferably 70, preferably 80% homologous to at least a

functional and/or specific part of the sequence of figure 6 or 7 or being at least 60, preferably 70, preferably 80% homologous to a protein encoded by at least a functional and/or specific part of the sequence of figure 4 or figure 5.

5 17. A method for inducing apoptosis in cells comprising providing said cells with Nmi/Hou-like protein activity and/or IFP-35-like activity together with apoptin-like activity.

10 18. Use of apoptin to find proteinaceous substances associated with apoptosis.

**ABSTRACT**

The invention relates to activation of apoptosis by means of interference of Hou-like and/or IFP35-like compounds.

Also the invention relates to anti-tumor therapies with compounds, which negatively interfere with Hou-like and/or IFP35-like compounds leading to induction of apoptosis, resulting in the elimination of tumor cells.

Also the invention relates to therapies for diseases related to aberrant apoptosis induction, such as auto-immune disease.

Also the invention describes the diagnosis of cells, which are susceptible to apoptin- or apoptin-like induced apoptosis.

GGGGGATCATGGAAGCTGATAAAGATGACACACAACAAATTCTTAAGGAGCATTTCGCCAGATGAA  
TTTATAAAAGATGAACAAAATAAGGGACTAATTGATGAAATTACAAAGAAAAATATTCAACTAAA  
GAAGGAGATCCAAAAGCTTGAAACGGAGTTACAAGAGGCTACCAAAGAATTCCAGATTAAAGAGG  
ATATTCCTGAAACAAAGATGAAATTCTTATCAGTTGAAACTCCTGAGAATGACAGCCAGTTGTCA  
AATATCTCCTGTTTCGTTTCAAGTGAGCTCGAAAGTTCCTTATGAGATACAAAAAGGACAAGCACT  
TATCACCTTTGAAAAAGAAGAAGTTGCTCAAAATGTGGTAAGCATGAGTAAACATCATGTACAGA  
TAAAAGATGTAAATCTGGAGGTTACGGCCAAGCCAGTTCCATTAAATTCAGGAGTCAGATTCCAG  
GTTTATGTAGAAGTTTCTAAAATGAAAATCAATGTTACTGAAATTCCTGACACATTGCGTGAAGA  
TCAAATGAGAGACAACTAGAGCTGAGCTTTTCAAAGTCCCGAAATGGGAGGCGGAGANGTGGAC  
CGCGTGGGACTATGACAGACAGTCCGGGAGTGCAGTCATCACGTTTGGNGGAGATTGGGAGTGGC  
TGACANNN

# Figure 1

Hou c6/#1

CGGAGTTACAAGAGGCTACCAAAGAATTCCAGATTAAAGAGGATATTCCTGAAACAAAGATGAAA  
TTCTTATCAGTTGAAACTCCTGANAATGACAGCCAGTTGTCAAATATCTCCTGTTTCGTTTCAAGG  
TGAGCTCGAAAGTTCCTTATGAGATACAAAAAGGACAATGCACTTATCACCTTTGAAAAAGGAAG  
AAGTTGCTCAAAATGTGNGTAANGCATGAGTAAACATCATGTACAGATAATAAGATGTAAATCTG  
GAGGTTACGGCCAAAGCCAAGTTCCATTAATATTCAAGGAGTCANGATTCCAGNGTTTATGCTAG  
AANGTTTCTAAAAATGANAATCAATGGTTACTGGAAATTCTGGACACATTGCGNTGAAAGATCA  
AGATGACGAAGACAAACTAAGAAGCTGAGCTTTTCAAAGTCCCGAAANATGGAAGAGCGGTTAGA  
GGGTGGNACCGCGTGNGANCTATGACAAGACAAGNCCGGGGAAGNTGCAGTCCATCACGTTTGTN  
NGAAGATTGGANGTNGGCTGACCAANGAATTTTAAAAAGGAGANGAATTACCCCTCTTTANGAG  
TAANATCAAAAACCCTGCCATAANAAGTTNACTGGTTTTCNCCCATTACACAGNAN  
TTACANNTTGANCAANANTANNCAGGATAATTTNCAGGGGAANAATCTNAAGNATGGCAAGNTGA  
CTTCTGGACAANGGT

**Figure 2**

Hou c17/#2



c6/#1	1	HEGRGI	MEADKDD	QILKEHSPDEFIKDEQNKG	LIDEITKKNIQLKKEIQKLETELQEA
Hou/Nmi	1	-----	MEADKDDT	QILKEHSPDEFIKDEQNKG	LIDEITKKNIQLKKEIQKLETELQEA
c6/#1	61		TKEFOIKEDIPETKMKFLSVETP	ENDSOLSNISCSFQVSSKVPY	EIQKGOALITFEKEEV
Hou/Nmi	55		TKEFOIKEDIPETKMKFLSVETP	ENDSOLSNISCSFQVSSKVPY	EIQKGOALITFEKEEV
c6/#1	121		AQNVVSMKHHVQIKDVNLEVTA	KPVPLNSGVR	FQVYVEVSKMKINVTEIPDTLREDQMR
Hou/Nmi	115		AQNVVSMKHHVQIKDVNLEVTA	KPVPLNSGVR	FQVYVEVSKMKINVTEIPDTLREDQMR
c6/#1	181		DKLELSFSKSRNGRRRCGP	RGTMTDSPGVQSSRLVEIGS	-----
Hou/Nmi	175		DKLELSFSKFRNGGGEV.D	RVDRQSGSAVITFVEIGV	DKILKKKEYPLYINQTCHRV
c6/#1	221		-----		
Hou/Nmi	234		TVSPYTEIHLKKYQIFSGT	SKRTVLLTGMEGIQMDEE	IVEDLINIHFQRAKNGGGEVDVV
c6/#1	221		-----		
Hou/Nmi	294		KCSLGQPHIAYFEE		

Figure 3

AGCAGGTGCTGCAACAAAAGGAGCACACGATCAACATGGAGGAGTGCCGGCTGCGGGTGCAGGTC  
CAGCCCTTGGAGCTGCCCATGGTCACCACCATCCAGGTGTCCAGCCAGTTGAGTGGCCGGAGGGT  
GTTGGTCACTGGATTTCTGCCAGCCTCAGGCTGAGTGAGGAGGAGCTGCTGGACAANCTANAGA  
TCTTCTTTGGCAAGACTAGGAACGGAGGTGGCNATGTGGACNTTCGGGANCTACTGCCAGGGANT  
GTCATGCTGGGGTTTGCTAGGGATGGAGTGGCTCANCCTGTGTGCCAAATCGGCCATTTACAGT  
GCCACTGGGTGGGCAGCANGTCCCTCTGAGAGTCTCTCCGTATGTGAATGGGGANATCCAGANGG  
CTGANATCAGGTCNCAGCCANTTCCCCGCTCGGTACTGGTGCTCAACATTCCTGATATCTTGGAT  
GGCCCGGAGCTGCATGACGTCCTGGANATCCACTTCCAGANCCCACCCGCGGGGGCGGAGATGT  
AAGACGCCCTGACAGTCGTACCCCAAGGACAACAGGGCCTAACAGTCTTCACCTCCTGAATCAAG  
GCTANGGGCCTCCCCCTTCTCATCCTCCCCACCCCCCGCCAAAGGTTCTCAANACTGGGCCTG  
GGCTTTNTG

#### Figure 4

IFP35 c14/#1

CCAAAGTGGCTGAGCAGGTGCTGCAACAAAAGGAGCACACGATCAACATGGAGGAGTGCCGGCTGCGGGTGC  
AGGTCCAGCCCTTGGAGCTGCCCATGGTCACCACCATCCAGGTGTCCAGCCAGTTGAGTGGCCGGAGGGTGT  
TGGTCACTGGATTTCTGCCAGCCTCAGGCTGAGTGAGGAGGAGCTGCTGGACAAGCTAGAGATCTTCTTTG  
GCAAGACTAGGAACGGAGGTGGCGATGTGGACGTTCTGGGAGCTACTGCCAGGGAGTGTCATGCTGGGGTTG  
CTAGGGATGGAGTGGCTCAGCGTCTGTGCCAAATCGGCCAAGTTCACAGTGCCACTGGGTGGGCANCAAGTC  
CCTCTGAGAGTCTCTCCGTATGTGAATGGGGAGATCCAGAAGGCTGAGATCAGGTTCGCANCCAGTTCCCCNC  
TCGGTACTGGGTGCTCAACATTCCTGATATCTTGGATTGGCCCGGAGCTGCATNACGTCCTGGANATCAACT  
TCANAAGCCCCACCCGCCGGGGCNGAGGTANAAGGCCTGACATCNTTACCCCAAAGGACAGCATGGNCCTA  
ACAGTCCTCACCTCCNAATCANGCTNNGGGGCTNCCCTTCTANCNTCCCCAACTG

**Figure 5**

IFP35 c33/#2

GGATCCACTGCCCTCTGCTTGCGGGCTCTGCTCTGATCACCTTTGATGACCCCAAAGTGGCTGAG  
CAGGTGCTGCAACAAAAGGAGCACACGATCAACATGGAGGAGTGCCGGCTGCGGGTGCAGGTCCA  
GCCCTTGGAGCTGCCCATGGTCACCACCATCCAGGTGATGGTGTCCAGCCANTTGAGTGGCCGGA  
GGGTGTTGGTCACTGGATTCCTGCCAGCCTCAGGCTGANTGAGGAGGAGCTGCTGGACAAGCTA  
TGAGATCTTCTTTGGCAANACTANGAACGGANGTGGCGATGTGGACGTTCTGGGAGCTACTGCCAG  
GGAGTGTGATGCTGGGGTTTGCTACGGATGGAGTGGCTCAGCGTCTGTGCCAAATCGGCCAGTTC  
ACAAGTGCCACTGGGTGGGCAGCAAGTCCCTCTGAGAGTCTCTCCGTATGTGANTGGNGAGATCA  
GAATGCTGANATTAAGTCGCATCCAATTCCTCGCTCNGGTACTGGTGCTCANNATCCTGANATCT  
TGGATTGGCCCCNGANTNCATGANATCTGGNAGATTCAATTNCANAAGTCCANCCNNCNGNGNCG  
GGAAGTANANGCCCGANANTTCNTNNCNTANGGNCAGCANNGCCTG

**Figure 6**

IFP35 c51/#3

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c33/#2      1 -----
IFP35       1 MSAPLDAALHALQEEQARLKMRLWDLQQLRKELGDS PKDKVPF SVPKIPLVFRGHTQQDP
c51/3#      1 -----

c33/#2      1 -----H GPKVAEQVLQQKEHTINMEECRLRVQVQPLELPM
IFP35       61 EVPKSLVSNLRIHCPLLAGSALITFDDPKVAEQVLQQKEHTINMEECRLRVQVQPLELPM
c51/3#      1 -----HEGRIHCPLLAGSALITFDDPKVAEQVLQQKEHTINMEECRLRVQVQPLELPM

c33/#2      37 VTTIQ..VSSQLSGRRVLVTGFPASLRLSEEEELLDKLEIFFGKTRNGGGDVDVRELLPGS
IFP35       121 VTTIQ..VSSQLSGRRVLVTGFPASLRLSEEEELLDKLEIFFGKTRNGGGDVDVRELLPGS
c51/3#      54 VTTIQVMVSSXLSGRRVLVTGFPASLRLXEEELLDKL*DLLWQXXERXWRC.....

c33/#2      95 VMLGFARDGVAQRLCQIGQVHSA GWASSPSES SVCEWGDPE -----
IFP35       179 VMLGFARDGVAQRLCQIGQFTVPLGGQQVPLRVSPYVNGEIQK EIRSQP PRSVLVLNI
c51/3#      104 .....PRSGATARECHAGVCYGW GSASVNP RP HKCHWVGSKE L*ESLR *XXRSEC*X

c33/#2      139 -----
IFP35       239 PDILGPELHDVLEIHFQKP ARGGGGR GPDSRTPTAGPSSLHL-----
c51/3#      156 *VAS SSLXYWCSXS*XLGL PXXMXS GRFNXXSPXXXXGKXXPXXSXXXXSXA

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Figure 7

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Filamin      1 RLRNGHVGISFVPKETGEHLVHVKKNGQHVASSPIPVVISQSEIGDASRVRVSGQGLHEG
c50/#1      1 -----
c57/#2      1 -----

Filamin     61 HTFEPAEFIIDTRDAGYGGLSLSIEGPSKVDINTEDLEDGTCRVTYCPTEPGNYIINIKF
c50/#1      1 -----
c57/#2      1 -----HEGRPTEPGNYIINIKF

Filamin     121 ADQHVPGPSFVSVKVTGEGRVKESITRRRRAPSVANVGSHCDLSLKIPEISIQDMTAQVTS
c50/#1      1 -----
c57/#2      18 ADQHVPGPSFVSVKVTGEGRVKESITRRRRAPSVANVGSHCDLSLKIPEISIQDMTAQVTS

Filamin     181 ESGKTHEAEIVEGENHTYCIREFVPAEMGTHTVSVKYKGQHVPGSPFQFTVGPLGEGGAHK
c50/#1      1 -----
c57/#2      78 ESGKTHEAEIVEGENHTYCIREFVPAEMGTHTVSVKYKGQHVPGSPFQFTVGPLGEGGAHK

Filamin     241 VRAGGPGLEPNEGVPEFS.EWTREAGAGLAHAVEEPKAEISFEDR.DSCGAYAV
c50/#1      1 -----
c57/#2     138 VRAGGPGLEKNE*EWSAEIRIQYEGPGKLVLEAWPELSXEPXLSLLRTATPVPVLMKV

Filamin     300 QEPGDYEVSVKFNEEHIPDSPFVVPVASPSGDARRLTVSSSLQESGLKVNQPASFAVSLNG
c50/#1      1 -----
c57/#2     197 XEPSE*XNPXQVSTKEHX-----

Filamin     360 AKGAIDAKVHSPSGALEECYVTEIDQDKYAVRFIPRENGVYLIDVKFNGTHIPGSPPKIR
c50/#1      1 -----
c57/#2     214 -----

Filamin     420 VGEFGHGDPGLVSAYGAGLEG.GVTGNPAEFVVNTSNAGAGALSVTIDGPSKVKMDCQE
c50/#1      1 -----HEGRGVTGNPAEFVVNTSNAGAGALSVTIDGPSKVKMDCQE
c57/#2     214 -----

Filamin     479 CPEGYRVTYTPMAPGSYLISIKYGGPYHIGGSPFKAQVTGPRLVSNHSLHETSSVFVDSL
c50/#1      42 CPEGYRVTYTPMAPGSYLISIKYGGPYHIGGSPFKAQVTGPRLVSNHSLHETSSVFVDSL
c57/#2     214 -----

Filamin     539 TKATCAPQHAGAPGPGPADASKVVAKGLGLSKAYVGOQSSFTVDCSKAGNNMLLVGVHGP
c50/#1     102 TKATCAPQHAGAPGPGPADASKVVAKGLGLSKAYVCEKSSFTVDCSKACITMLLVGVHGP
c57/#2     214 -----

Filamin     599 TPCEILVKEVGS.RLYSVSYLLKDKGE.YTLVVKWGEHHPGSFYR.VVP-
c50/#1     162 TPCEILVKARGQPALQRVLTCPKDKGEVHTGGQNGGDYQIECKPLP.CGCP
c57/#2     214 -----

```

Figure 8